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## INTRODUCTION

More than 70% of breast cancer patients show bone metastases at the time of autopsy. Bone metastasis is a devastating symptom, which can cause bone fractures, severe pain, hypercalcemia and/or neurological paralysis. Despite its clinical importance, the mechanisms that cause preferential bone metastases are not well understood. Currently no efficient methods to prevent or treat bone metastases are available. There have been two major concepts to help to understand organ preferential metastasis: (a) cancer cells proliferate in a desirable environment ("seeds and soil theory") (Paget, 1889) and (b) cancer cells in the circulation are physically trapped in the first downstream organ (Ewing, 1928). Bone metastasis of breast cancer is likely to be caused by multiple factors related to these two classic concepts. However, we are unable to explain why some breast cancers metastasize to bone at an early stage, and why some breast cancers metastasize to bone but not to the lymph nodes. The most common site for bone metastasis in breast cancer is the spine, which is, however, not the first organ downstream of the breast. We have hypothesized that breast cancer cells released into the circulation from the primary organ adhere to the cells in bone marrow through receptor/ligand-like specific interaction(s). To test this hypothesis, we have used two approaches; [1] to identify specific peptide motif(s) that home to bone by using phage libraries of random peptides, and [2] mammalian expression cloning to identify proteins that are expressed on the surface of breast cancer cells and that might confer bone homing.

## BODY

### **To identify specific peptide motifs that home to bone by using random peptide libraries in phage**

We have used libraries made with fD phage, which we obtained from Drs. Ruoslahti and Pasqualini at our Institute, and commercial libraries made with fD phage (New England Biochem, ME) containing both linear and loop-type peptides.

Initially we used a mixture of 7 random peptide libraries, CX5C, CX6C, CX7C, CX9, X4YX4, CX3CX3CX3C, CX3CX4CX2C, which had proven of good quality for *in vitro* panning in the hands of our collaborators. Ten to the eleventh units of the library mixture was injected into the tail vein of 7-week old female Balb/c mice and allowed to circulate for 3 min. After snap freezing the mice in liquid nitrogen, bone marrow tissue was collected by flushing out the femurs, washed and incubated with host *E. coli*. A total of 160 single colonies of infected bacteria were isolated, expanded individually and then pooled. The phage were purified and re-injected. We repeated 3 cycles of injection and recovery of phage. Two clones appeared 3 times and one clone appeared twice in 47 clones sequenced. We continued to run 3 additional cycles of injection and recovery and obtained sequences of 36 additional clones. We also performed three additional cycles using perfusion instead of snap freezing in order to minimize the destruction of the tissues and the possible leakage of phage into the extra vascular cavity in the bone marrow and sequenced an additional 36 clones. In a second set of experiments, the slightly simpler libraries, CX6C, CX7C and CX10C, were mixed with non-infective fUSE5 to block non-specific interaction. The mouse was incubated for 60 min. and then perfused. After 3 cycles of injection and recovery from about 280 single clones, 4 clones appeared twice in 35 sequenced. In both sets of experiments, the enrichment of clones was not as high as we had originally expected. We found that bone marrow



tissue retained an extremely high number of phage compared to the numbers per weight in other organs, such as brain, kidney and lung indicating that there is non-specific capturing of phage in the bone marrow.

### **Establishment of immortalized bone marrow cells**

As a complementary approach to *in vivo* phage display, we decided to include panning *in vitro* using bone marrow stromal cells, since interaction between bone marrow stromal cells and several types of bone metastatic cancer cells (melanomas, prostate cancer, etc) have been reported. Since it is not known which cell type may play an important role in the initial settlement of breast cancer cells in bone tissue, we focused on both endothelial and stromal cells as putative candidate cell types.

Primary cultures of bone marrow cells contain predominantly endothelial cell-like populations, when medium supplemented with endothelial cell growth factor (ECGF) is used. However, if they are cultured without ECGF, large flat cells are clearly observed, and hematopoietic cells reside on the top of these large cells, suggesting that these primary cultures contain stromal cells. In order to obtain both stromal and endothelial populations that may interact with breast cancer cells bone marrow tissue was collected from tibias and femora of four 10-week-old female Immortomice (Charles River, MA) and digested with collagenase A. The Immortomouse is a transgenic mouse carrying a temperature sensitive SV40 large T antigen under control of the mouse major histocompatibility complex H-2Kb promoter. One half of the bone marrow cells were cultured without ECGF, while the other half were cultured with ECGF to obtain endothelial cell clones. All the cells were maintained with 100 units/ml  $\gamma$ -interferon in order to activate the H-2Kb promoter, and incubated at 33°C which was permissive condition for the temperature sensitive large T antigen. After two passages, single-cell cloning was performed by the limiting dilution procedure. Four to five weeks later, six clones cultured without ECGF (stromal cell group) and five clones cultured with ECGF (endothelial cell group) were trypsinized and expanded. After single-cell cloning of the primary culture at permissive conditions, seven stromal cell clones and nine endothelial cell clones were obtained, and their morphology and the expression patterns of marker proteins were examined (Table1)

### **Development of a binding assay**

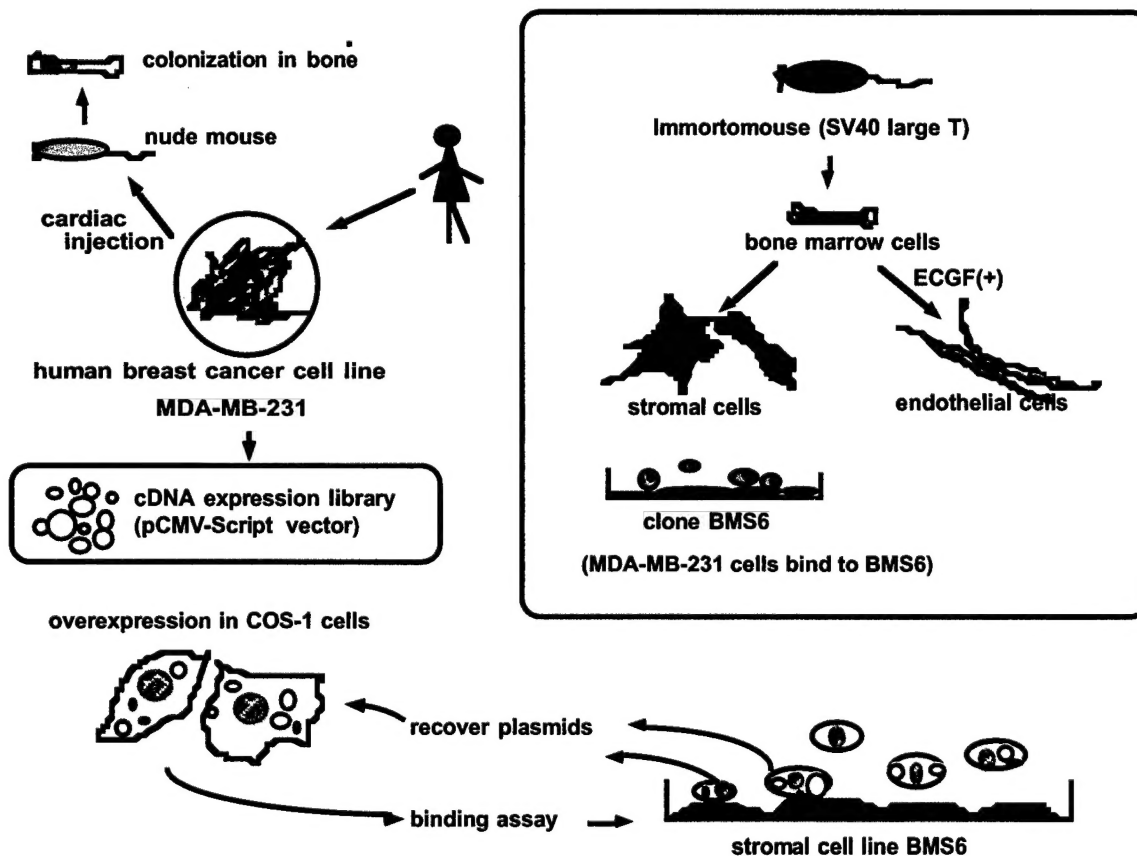
The human breast cancer cell line MDA-MB-231 is believed to maintain "bone metastatic characteristics", since it can colonize the bone marrow of nude mice if injected through the left cardiac ventricle, while the control breast cancer cell line MCF7 cannot (Yoneda et al., 1994). We previously observed that MDA-MB-231 cells contained a subpopulation that bound to primary cultures of mouse bone marrow cells.

Thus, we tested the ability of each of the newly immortalized cell lines to bind to the metastatic breast cancer cell line MDA-MB-231 and determine which of the immortalized cell lines would be most useful in developing a panning assay. For this we focused on two different designs of the binding assay.

**Table1**

	clones	expression (immunostaining)					binding assay				
		AP	VWF	VCAM-1	F4/80	CD44	MB231 disk	MB231 reg.	MB361 reg.	MCF7 reg.	COS-1 reg.
stromal -like	BMS1	+		-		+++	+	++			
	BMS2	+		-		+	+ -	-			
	BMS3	+++		-			++	+++			
	BMS4			-		++					
	BMS5	+++		-		++	+ -	+ -			
	BMS6	+		-	-	++	+++	+++	-	-	-
	BMS8	+++		-		+	-	+ -			
endothelial -like	BME1	-					-				
	BME2	-					-				
	BME3		++	++							
	BME4							+			
	BME5	+	++	+++	-		++	+	-	-	-
	BME6	-					-	++			
	BME7						+++				
	BME8							++			
	BME9	++					-	+++			

## Strategy of the Expression Cloning



**Fig. 1** Bone marrow cells were isolated from Immortomouse and clone BMS6 and BME5 were chosen due to their ability to capture MDA-MB-231 cells. cDNA expression library of bone metastatic breast cancer MDA-MB-231 was expressed in COS-1 cells. Cytoplasmic DNA from the COS-1 cells bound to BMS6 was recovered and this cycle was repeated.

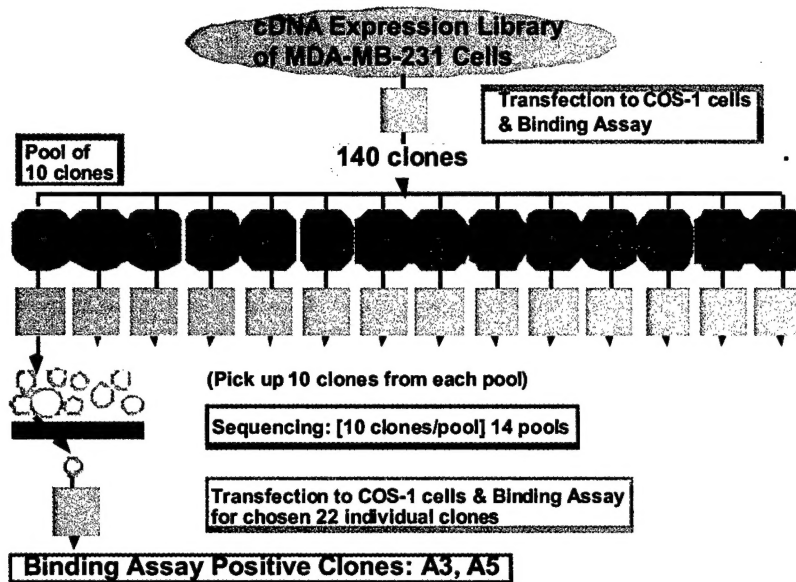
One is an ordinary binding assay whereby the stromal cell lines or endothelial cell lines are coated on a microtiter plate and the MDA-MB-231 breast cancer metastatic cell line is then added to the plates and binding to the bottom of the plate measured with time. The problem with this binding assay is that the unspecific binding of breast cancer cell lines to the stromal and/or endothelial cell lines is high. Therefore we developed a novel assay using a plastic disc that could be coated with the stromal or endothelial cell lines. This disk is then floated on top of medium containing suspended MDA-MB-231 breast cancer cells. This forces the cancer cells to migrate in liquid solution against gravity towards the disc containing the stromal or endothelial cells. We have observed that this binding assay gives very low background. Based on this assay, we chose to use the clone BMS6 as a stromal cell clone since MDA-MB-231 cells bind well in both assay systems and BMS6 cell line expresses the stromal cell line marker alkaline phosphatase. We also chose BME5 as the endothelial cell clone because of their binding to MDA-MB-231 as well as the expression of endothelial cell markers AP, von Willebrand factor (vWF) and VCAM-1. BMS6 or BME5 did not show binding to other types of breast cancer cells such as MCF-7 and MDA-MB-361. MCF7 cells do not colonize in nude mice and MDA-MB-361 are derived from brain metastasis not bone metastasis.

Next, in order to complete the set of reagents needed for the cloning strategy as depicted in Fig. 1, we constructed a cDNA library of MDA-MB-231 cells using a mammalian expression vector, pCMV-Script. This vector carries an SV40 origin of replication, which allows episomal replication in cells that express the SV40 large T antigen, such as Cos-1 cells. This plasmid library has an average insert size of 1.3 kb and a size of  $4.0 \times 10^5$  primary colony forming units.

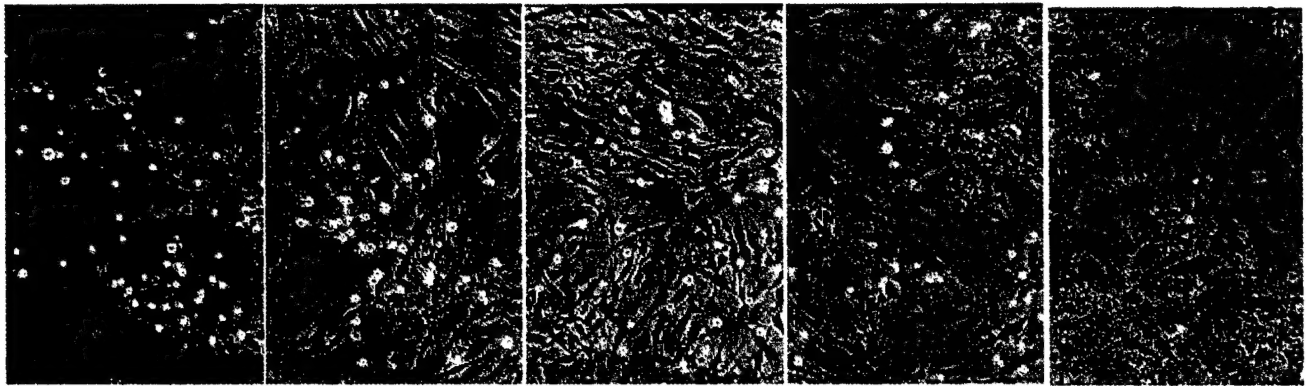
### **Expression Cloning with BMS6 cells**

Forty  $\mu$ g plasmid DNA encompassing the entire library were transfected into COS-1 cells by electroporation, and the cells were incubated with ethanol-fixed BMS6 cells. Amplified DNA was recovered from the cytoplasm of bound COS-1 cells and used for transformation of XL-10 Gold Ultracompetent Cells (Stratagene, La Jolla, CA). A total of 140 clones were collected after the first cycle, cultured individually, and divided into ten groups (A to N) as depicted in Fig. 2. Fifty  $\mu$ g of DNA from each group were transfected into COS-1 cells again, and amplified plasmids from the bound COS-1 cells were recovered. Ten cDNA clones from each group were sequenced, and the sequences were compared to the databases. Twenty-two clones were chosen because they appeared more than twice, had large insert (more than 1 kb), were unknown sequences, or were membrane proteins. Each cDNA clone was individually transfected into COS-1 cells. Clone A3 and A5 showed significant binding, and clone H7 was considered negative as shown in Fig. 3.

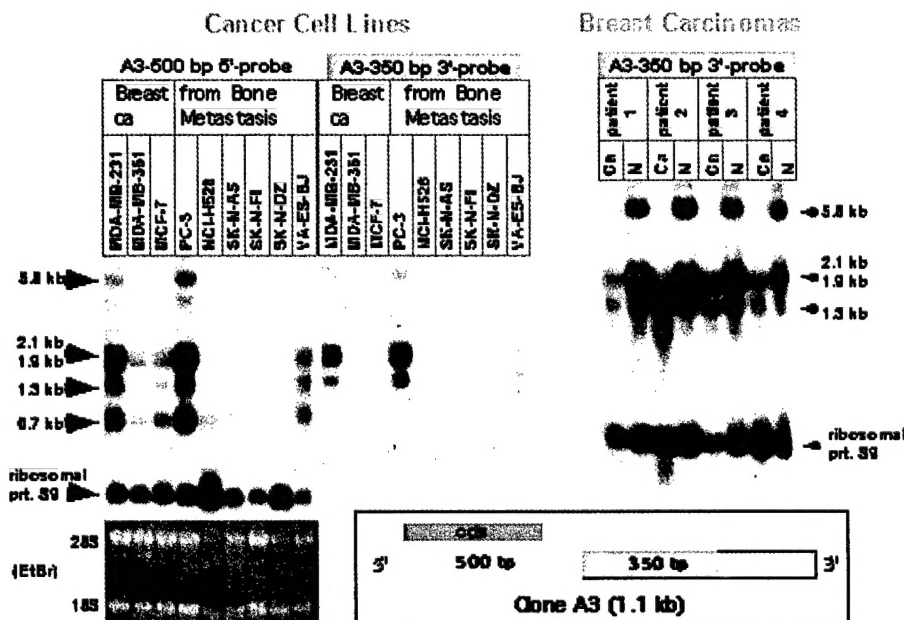
Clone A3: Clone A3 is a 1.1 kb fragment containing entire coding sequence of CD59, which is known as a GPI anchored membrane protein. CD59 binds to complement 8 and 9. It is suggested that CD59 expressed on the surface of cancer cells protects them from complement attack (Chen et al., 2000), although no function for CD59 has been proposed in cell-cell adhesion. Northern blot hybridization with a probe of clone A3 to human cancer cell lines is shown in Fig. 4. Clone A3 is highly expressed in MDA-MB-231 and a prostate cancer cell line PC3, which was isolated from bone metastasis. The expression of clone A3 is lower in MDA-MB-361 cells, which are a breast cancer line isolated from brain metastasis, and breast cancer cell line MCF7, which does not colonize the bone marrow in nude mice (Yoneda et al., 1994).



**Fig. 2** Recovered 140 clones were divided into 10 groups. DNA of each group was transfected into COS-1 cells again, and 10 clones from each group were analyzed. Twenty two clones were individually transfected, and binding was compared.



**Fig. 3** Binding assay. COS-1 cells transfected with clone A3, A5, and H1, incubated with fixed BMS6 cells. MDA-MB-231 cells were positive control



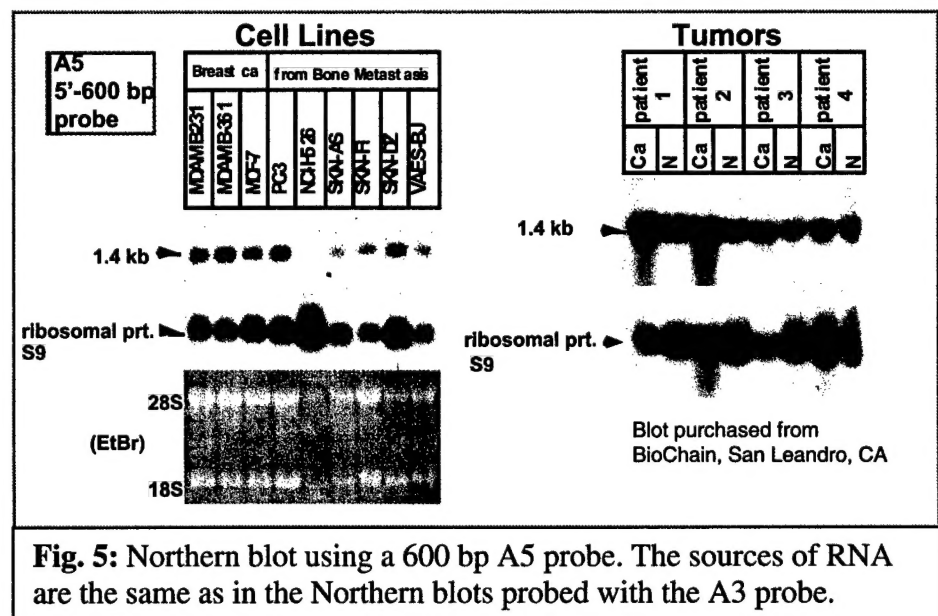
**Fig. 4.** Northern blots with A3 (CD59) probes. MDAMB231, MDAMB361, MCF7(breast cancer lines). PC3 (prostate cancer line). NCI-H525 (lung cancer derived from bone metastasis). SK-N-AS, SK-N-FI, SK-N-DZ (sarcoma lines derived from bone metastasis).

The remaining cell lines analyzed were non-breast cancers but isolated from bone metastasis. The multiple bands seen in each lane as shown in Fig. 4 are known to be due to alternative poly-A adenylation (Tone et al., 1992). This result suggests that CD59 can be a candidate molecule that mediates bone preferential homing of breast cancer. Interestingly, expression level of cD59 in primary breast cancer tissue is lower than normal mammary gland of the same patients (Fig.4, right). This may suggest that function of CD59 in normal mammary gland is different from that in cancer cells.

To test if CD59 actually confers cell adhesion a cell attachment assay was performed. Flat-bottom ELISA plates were coated with 10 µg/ml rabbit anti-mouse IgG (Dako, Denmark). Monoclonal antibodies against CD59, 193-27 (NeoMarkers, Fremont, CA) and MEM-43 serially diluted in 1% BSA-PBS were incubated in the coated wells at 37 °C for 2 hrs. Human cancer cell lines were harvested with 3 mM EDTA -PBS, and 100 µl of  $3.2 \times 10^5$  cells/ml suspension was added into each well. The plate was incubated at 37 °C in 7% CO<sub>2</sub> incubator for 1 hr, and non-attached cells were removed by washing with PBS. The plate was fixed with 2.5% glutaraldehyde in PBS for 30 min, and then stained with crystal violet (Fig. 5). MDA-MB-231 cells and PC3 cells were captured by the anti-CD59 antibodies and stained positive, while K562 (human leukemia line) and COS-1 cells were negative. Adhesion was not observed with unrelated control monoclonal antibodies against tumor markers (MSN1 and SSEA-1).

In order to perform *in vivo* experiments to test the homing of cells expressing CD59, we made CHO (Chinese hamster ovary) cells expressing both CD59 and a marker enzyme (placental alkaline phosphatase, PLAP) as well as CHO cells expressing only PLAP as a control. Expression levels of CD59 in the CHO cell clones were low according to the results of immunohistochemistry which showed low frequency of positive cells. We performed single cloning from the best positive CHO clone, but the frequency of positive cells in these sub-clones were the same as the original clone. The CHO clones expressing low levels of CD59 did not show binding to BMS6 cells *in vitro*. We also tested if these clones preferentially home to bone *in vivo* when injected into mice as shown in Fig. 6; however, so far we have been unable to detect bone homing with the CD59-expressing cells.

**Clone A5:** The sequence of clone A5 (1.0 kb) is highly homologous to a previously cloned cDNA deposited in the GenBank; however, 5' end of A5 differs from this cDNA, and A5 lacks a 66 bp short sequence in the middle. We confirmed existence of A5 transcript by RT-PCR using specific primers, and hypothesize that A5 is a splicing variant of this known sequence.





The results of Northern blot hybridization with a probe, which would recognize both A5 and the known sequence, in human cancer cell lines and human breast cancer tissues are shown in Fig. 5. This probe was positive in all the cancer cell lines except a lung cancer cell line. Non-bone metastatic breast cancer cell lines (MDA-MB-361 and MCF7) are also positive. Expression in human breast cancer tissues is higher than in normal control tissues in at least in two of the four cases, although the RNA samples in this particular commercial blot show some degradation. Work continues on this clone.

### **In vivo homing after cardiac injection**

In our initial proposal, we had planned to isolate peptide motifs that home to bone marrow by *in vivo* organ targeting strategy. Despite the problems encountered with non-specific phagocytosis, we still believed that an *in vivo* system might be advantageous and worth testing with a new *in vivo* expression cloning as shown in Fig. 7. In this approach, COS-1 cells expressing cDNAs from MDA-MB-231 were prepared in the same protocol as *in vitro* cloning except that the suspension of COS-1 cell was strained with 40  $\mu$ m nylon mesh. COS-1 cells transfected with each cDNA ( $5 \times 10^6$  cells) were injected into the left cardiac ventricle of a female mouse which were anesthetized with intraperitoneal injection of Avertin. One hour later, injected mice were euthanized with CO<sub>2</sub> gas and bone marrow from hind limbs and approximately 100 mg of lung tissue were collected from each mouse. Plasmid DNAs in COS-1 cells were recovered from the bone marrow tissue of injected mice by a modified Hirt method (Arad, 1998). We have obtained total 20 cDNA clones (three genes were obtained twice) as shown in below (Table 2).

We have chosen 15 clones (bold letters in Table 2) for individual analysis. COS-1 cells expressing each clone was injected into mice together with COS-1 cells transfected with a plasmid pSVT7, which carries an ampicillin resistant gene. Fifty % of the bacteria were spread on tetracyclin plates, which allow bacteria cells transformed with the cDNA in pCMV-Script, and the other 50 % were used to transform ampicillin plates, which allow bacteria cells transformed with the control pSVT7. COS-1 cells transfected with pSVT7 do not express any gene from the library, and the number of colonies in the ampicillin plates are considered nonspecific background. The ratio of kanamycin resistant bacteria colonies, which contain the testing cDNA clones in pCMV-Script, and the number of ampicillin resistant bacteria colonies, which contain pSVT7, were compared between the DNA samples recovered from the bone marrow and lung. Since the lung traps COS-1 cells randomly, we use lung sample as control tissue. Out of the 15 clones, only one clone, 11.4 showed positive results. The KM:Amp numbers were 14:9 in the first experiment, and 6:0 in the second experiment. The clone 11.1 is a cDNA originally isolated from human testis and encodes a hypothetical protein FLJ10468. This cDNA clone 11.1 should be tested with Northern blots and the stable transformant of the clones need to be tested for bone homing using the similar experiment as described in the earlier paragraph.

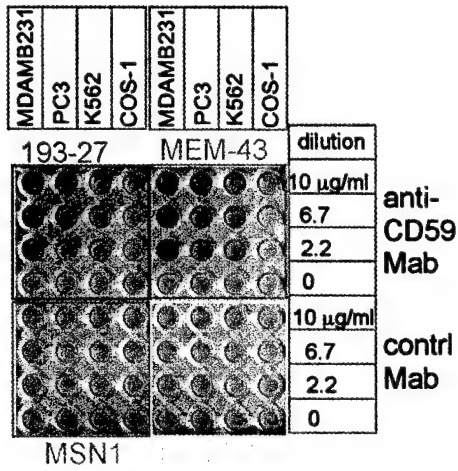


Fig. 6: Cell attachment assay

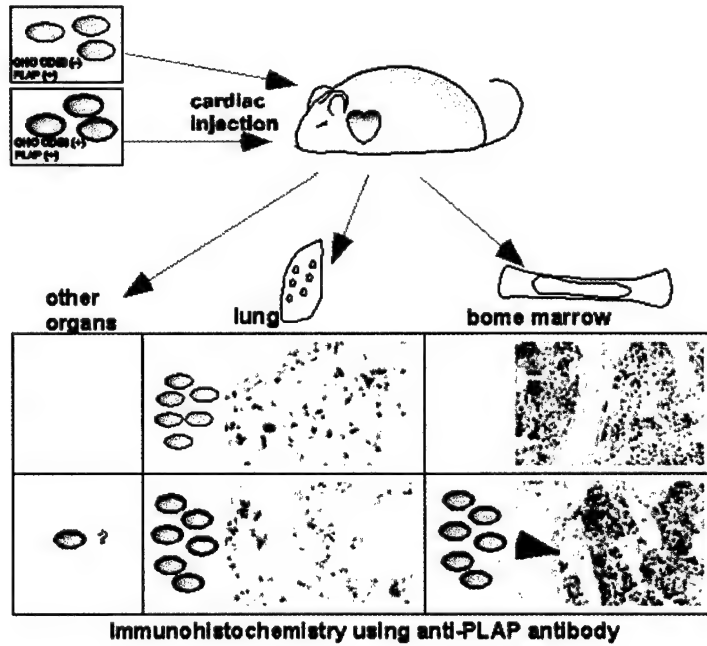
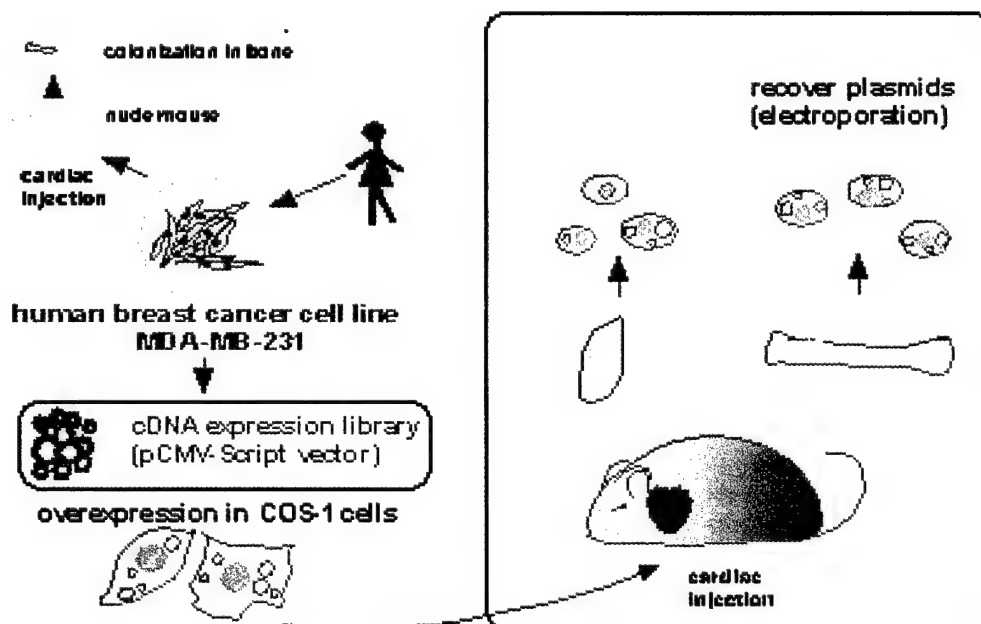


Fig. 7: Method to test bone homing.

Fig. 8: A new in vivo expression cloning system



clone	bac	size	Blast nr	Blast est	known prt?	predicted prt	source	chr
26.1	XL10	2.7	AC008114 (BAC)	BE889643	no	?	leiomyosarcoma	12
26.3	XL10	1	AK001750			FLJ10888	terato ca	4
26.4	XL10		AF347002		mitochondrial DNA			mito
11.1	XL-B	1.5	XM_002665.6	BF593640	Cys-rich protein		moter neuron, glioblastoma	4
11.2	XL-B				ribosomal protein S30		hypothalamus	11
11.4	XL10	1.8	XM_028947.1	BI463917		FLJ10468	testis	1
11.6	XL10	0.85*	XM_004593.1	BI115910	sorcin (ca binding prt)		lung small cell ca, MGC3	7
11.7	XL10		BC005002	BI834883	mitochondria ribosomal		lymphoma,pancr.spleen	5
11.8	XL10	0.35	AC092849.4	AI524014	no	ORF 155 aa	B-cell leukemia	7
11.9	XL10	1.3	BC014391	BI911460	no	ORF 219 aa	leukocyte, renal ad.ca	2
11.10	XL10	2.0**	XM_048403.1	BI011099	filamin A, actin binding prt		normal lung,	X
11.11	XL10	1.6	BC001357		no	SP260, FLJ10243	melanoma	
11.12	XL10	0.65	XM_001468		calpactin I(p11)ca-binding			1
11.13	XL10	0.28***	?		unknown			1
11.14	XL10	0.65	XM_001468		calpactin I(p11)ca-binding			
11.15	XL10	1.5	XM_002665.6		Cys-rich protein		moter neuron, glioblastoma	4
11.16	XL10	1.7	BC001590		man.6 P receptor binding prt		colon adeno ca	19
11.17	XL10		AB009010		ubiquitin C			
11.18	XL10	0.28***	?		unknown			12
11.19	XL10	1.4	NM003299.1		tumor rejection antigen(gp96)		terato ca LASTD	
11.20	XL10	0.6	XM_03470.1		DAD-1		Raji	

\* 0.4+0.45

\*\*1.4+0.6

\*\*\*0.18+0.1

Table 2

## KEY RESEARCH ACCOMPLISHMENTS

- \* Establishment and characterization of bone marrow stromal and endothelial cell lines
- \* Development of two binding assays to be used in expression cloning by an *in vitro* panning procedure
- \* Cloning and initial characterization of two novel cDNAs.
- \* Development of an *in vivo* screening procedure for organ-specific targeting
- \* Identification and cloning of clone FLJ10468 coding for a putative protein of unknown function.

## REPORTABLE OUTCOMES

A manuscript describing these novel panning and *in vivo* targeting strategies is almost ready for submission. A copy will be forwarded.

Our results have been presented at the following conferences:



- Era of Hope (Department of Defense Breast Cancer Research Program Meeting), Atlanta, GA June 2000. Oral presentation of abstract entitled: "Homing of breast cancer cells to bone marrow". Proceedings Vol. II, 583 (2000)

- The 28<sup>th</sup> Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM), September 8-13, 2000 in Munich, Germany. Oral presentation of abstract: "Strategy to clone molecules mediating metastasis of breast cancer cells to bone". Tumor Biology 21: S1, pg 64, 2000.

- 92nd Annual Meeting of American Association for Cancer Research, New Orleans, LA, 27 March 27, 2001. Poster entitled "Strategy to clone molecules mediating metastasis of breast cancer to bone. AACR Proceedings 42, 793 (2001).

## CONCLUSIONS

The work funded by this research grant has allowed us to test the robust technique of *in vivo* targeting to the problem of bone marrow homing and the development of bone metastasis. The use of this approach, however, has proven to be very unreliable due to major difficulties with nonspecific binding and phagocytosis of phage in the bone marrow. Nevertheless, alternative an *in vivo* panning strategy has been developed. This grant has also made possible the generation of very valuable cell lines of endothelial and stromal cell origin and the development of a novel *in vitro* binding assay. These new reagent and methodologies will be made available to the research community at large to facilitate further advances in the area of bone metastasis. Using these new reagents and methodologies we have cloned a number of novel cDNAs, which require further study. While our project has not completed all the stated goals, the work has progressed sufficiently so that competitive grant proposals can now be sent to the National Institute of Health, USA for consideration for funding and further studies on these newly uncovered molecules.

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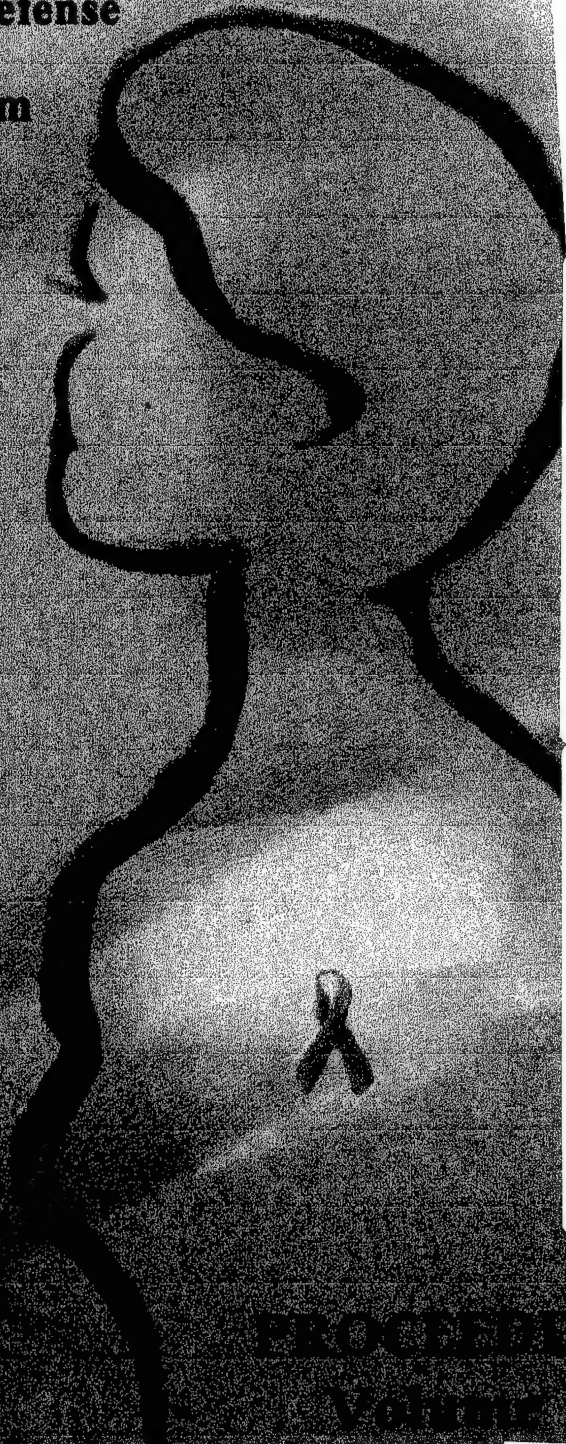
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# *Era of Hope*

Department of Defense  
Breast Cancer  
Research Program  
*Meeting*



PROCEEDINGS  
Volume II

## HOMING OF BREAST CANCER CELLS TO BONE MARROW

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Approximately 70% of breast cancer patients show bone metastasis at the time of autopsy. The presence of bone metastasis significantly lowers the quality of life of these patients. In spite of its clinical importance, the mechanisms of bone metastasis are poorly understood. Our work focusses on the initial settlement of breast cancer cells in the bone marrow as we hypothesize that breast cancer cells released from the primary tumor adhere to stromal and/or endothelial cells in the bone marrow through specific receptor/ligand-like interactions. Our aim is to identify, clone and characterize the molecules that may mediate these interactions.

We are using a bone metastatic human breast cancer cell line, MDA-MB-231, specifically a subpopulation that binds to primary cultures of adherent bone marrow cells. We have established immortalized cell lines from the bone marrow of the "Immortomouse" and characterized a group of cell lines that display bone marrow stromal cell-like phenotype, and another with properties of bone marrow endothelial cells based on morphology and marker expression. One cell line from each group was chosen based on their strong binding to the MDA-MB-231 cells in order to develop binding assays to be used to identify putative clones. We have constructed a cDNA library of MDA-MB-231 cells using a mammalian expression vector pCMV-Script. COS-1 cells transfected with the cDNAs of this library were incubated with the stromal cell clone, BMS6, and the plasmid DNAs were recovered from the bound COS-1 cells. The clones were expanded individually, and pools of 10 clones each were retransfected into COS-1 cells. This approach has yielded four known molecules, three known DNA sequences, and three unknown DNA sequences. We are currently obtaining full-length cDNA clones of these candidates to test them individually our binding assay. The same strategy will also be applied for the immortalized endothelial cell clone. We plan to use a mouse model system in order to test if the candidate molecules cause bone homing *in vivo*.

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# Tumor Biology

Tumor Markers and Translational Cancer Research

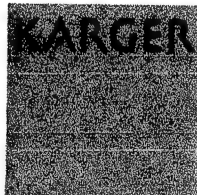
**Abstracts**



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**From Tumor Biology  
to Clinical Oncology**

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**PROGNOSTIC MARKERS IN BREAST CANCER BEFORE AND AFTER INTENSIVE OR HIGH DOSE CHEMOTHERAPY****Sabine Kasimir-Bauer<sup>1</sup>, S Mayer<sup>1</sup>, P Bojko<sup>1</sup>, D Borquez<sup>1</sup>, R Neumann<sup>2</sup>, S Seeber<sup>1</sup>****<sup>1</sup>Innere Klinik und Poliklinik (Tumorforschung), Westdeutsches Tumorzentrum, Universitätsklinik Essen, <sup>2</sup>BayerAG, Krefeld, Germany**

We monitored micrometastases in the bone marrow (BM) and peripheral blood stem cells (PBSC) of patients (pts) with locally advanced (group 1; n=13) and metastatic breast cancer (group 2; n=30). Cytokeratin (CK) and 17-1A-positive cells in the BM as well as tumor markers (TM) in blood (CEA, CA-15-3, TPA, c-erbB-2) were studied by immunocytochemistry (pan CK antibody A45B-B3), luminometric assays and ELISA. Before therapy, CK+ cells could be detected in the BM of 3/13 (23%) group 1 pts and in 18/30 (60%) group 2 pts, respectively. PBSC samples were positive in 2/9 (22%) group 1 pts and in 7/24 (29%) group 2 pts. After chemotherapy, CK+ cells were only detected in the BM of group 2 pts. CK and 17-1A+ cells could be detected in both pt groups prior to therapy, in PBSC samples and in group 2 pts after therapy. High values for TPA and c-erbB-2 were only detected in group 2 pts. In conclusion, the analysis of BM and blood is useful to determine residual tumor load and the identification of immunotherapeutic target antigens on CK+ cells may help to stratify pts for additional biological therapies.

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**STRATEGY TO CLONE MOLECULES MEDIATING METASTASIS OF BREAST CANCER CELLS TO BONE****Sonoko Narisawa and JL Millán****The Burnham Institute, CA, USA**

Our work focuses on the initial settlement of metastatic cancer cells in bone. We hypothesize that breast cancer cells released from the primary tumor adhere to stromal and/or endothelial cells in the bone marrow through specific receptor/ligand-like interactions. The human breast cancer cell line, MDA-MB-231 was chosen as our model of bone metastatic breast cancer, and using the "Immortomouse" we established and characterized cell lines that display either bone marrow stromal or endothelial cell phenotypes. One cell line from each group displaying significant binding to the MDA-MB-231 cells were chosen for the development of a binding assay to be used to identify putative homing molecules. We constructed a cDNA library of MDA-MB-231 cells using a mammalian expression vector, pCMV-Script. COS-1 cells transfected with the cDNA library were incubated with the immortalized cell lines and the plasmids were recovered from the bound COS-1 cells. Two cDNAs, A3 and A5, were identified as conferring significantly binding in our assay. By Northern blot, A3 was expressed in higher amounts in bone metastatic tumor cell lines while A5 appeared to be over-expressed in breast and prostate cancer cell lines irrespective of their metastatic potential.





*American Association for Cancer Research*

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#### #4253 Evaluation of Concordance Between Bone Histology and Technetium-99 Methylene Diphosphonate Bone Scan in Advanced Prostate Cancer Patients with Special Reference to Bisphosphonate Treatment. Martine P. Roudier, Celestia Higano, Lawrence True, Susan Ott, David Penson, Robert Vessella, and Paul Lange. *University of Washington, Seattle, WA.*

Bone metastases of prostate cancer are typically osteosclerotic. There is experimental, histological and biochemical evidence of increased bone resorption, which correlates with bone pain. These facts provide the rationale for the use of bisphosphonates (BPs) in managing patients with skeletal metastases of prostate carcinoma. BPs selectively link to sites of active bone remodeling, where they are potent inhibitors of osteoclast-mediated bone resorption. The high avidity of BP for calcium ions delivered by osteoclasts is the basis of their use as scanning agents when coupled with a  $\gamma$ -emitting radioisotope such as technetium. Consequently, BP treatment and Technetium-99 methylene diphosphonate (Tc99 MDP) might be in conflict when visualizing the localization of bone metastases. This study evaluated the concordance of histology and bone scans in the diagnosis of bone metastases in 11 patients who died with advanced prostate cancer. Five of them were treated by BP 3 to 21 months before death. Two treated patient samples are in process. Using formalin fixed paraffin embedded tissue from 6 to 25 separate bone sites from each of 11 patients in our Prostate Cancer Donor Program, we assessed the concordance of findings between the histology and the bone scans (between 1 and 10 bone scans per patient) from each of the patients. Five patients had repeated biopsies at each bone site - for histology, - for histomorphometry. Overall correlation between histology and bone scans was 85 % in non-treated patients and 35 % in BP treated patients. In one patient, using Tc99 MDP, 10 repeated bone scans have not been able to detect 14 separate bone metastases that were found histologically. These 14 bone metastases were associated with no measurable histomorphometric changes, which confirms the bone scan data. In one other patient, 5 bone scans revealed more than 19 bone metastases; none of these were histologically demonstrable. In the other patient, there was 100 % correlation between histology and bone scan. This patient was treated with BP for 3 months; sites involved with cancer were seen on the bone scans taken 4 years before treatment. He had diffuse osteoblastic metastases. In this study, Tc99 MDP bone scan has been shown to be less than 100 % sensitive in detecting bone metastasis in BP-treated patients. Especially in long-term BP treatments of prostate cancer, BPs may negatively impact the detection of metastases by bone scans but may favorably impact the course of cancer progression.

#### #4254 Dipeptidyl Peptidase IV (DPPIV) Inhibits Cellular Invasion of Melanoma Cells. Charit L. Pethiyagoda, Danny R. Welch, and Timothy P. Fleming. *Pennsylvania State University School of Medicine, Hershey, PA, and Washington University School of Medicine, St. Louis, MO.*

Metastasis of tumor cells to distant organs remains the primary cause of cancer fatalities. Yet, the number of genes that have been identified as playing a major role in the dissemination of tumor cells to distant organs lags far behind the array of genes that have been implicated in the tumorigenic process. Dipeptidyl Peptidase IV (DPPIV) is a 110kD, trans-membrane, mainly extra-cellular glycoprotein, with unknown function. Constitutively expressed on numerous epithelial cell types, DPPIV is often dysregulated in a variety of human malignancies. The most striking evidence of DPPIV down-regulation is found in transformed melanocytes, where nearly 100% of melanomas lack DPPIV expression. We have identified DPPIV as a gene that can alter the invasive potential of a number of melanoma cell lines. By transfecting the full-length cDNA of DPPIV, we have established stable melanoma cell lines that express comparable levels of the DPPIV protein as normal epidermal melanocytes. Matrigel invasion assays were utilized to study the effects of DPPIV expression on the invasive potential of these cells. The parental and vector transfectants readily migrated across the Matrigel while the invasiveness of DPPIV transfected cells was reduced by greater than 75% in at least two independent melanoma cell lines. The effects on cellular invasion are not attributed to overall growth characteristics, as both DPPIV expressing and non-expressing cells behave comparably in culture and form tumors in-vivo. We have also constructed mutants of DPPIV that lack either the extra-cellular serine protease activity or the six amino acid cytoplasmic domain. Both mutants were stably expressed in melanoma cells. Matrigel invasion assays performed with cells expressing the two mutant forms of the protein revealed phenotypic effects similar to wild type function. Therefore, in this study, we have demonstrated that expression of a proteolytically active form of the DPPIV protein inhibits the invasiveness of malignant melanoma cell lines lacking endogenous DPPIV expression. Furthermore, we have shown that neither the protease activity nor the cytoplasmic domain of DPPIV is required for its anti-metastatic activity.

#### #4255 Regulation of the Outcome of Intravascular Cancer Cells in the Liver by Lipopolysaccharide (LPS). F. William Orr. *University of Manitoba, Winnipeg, MB, Canada.*

Our previous studies showed that hepatic microvascular adhesion molecule expression and nitric oxide (NO) mediated tumor cell cytotoxicity are inducible by microenvironmental factors and are variably expressed in different zones in the liver acinus. Here we present more evidence that the hepatic microvasculature regulates the fate of metastatic melanoma cells in the liver. After injection of fluorescently labeled melanoma cells ( $5 \times 10^5$  cells / 0.15 ml) into a mesenteric vein of the C57BL/6 mouse, double-labeling immunohistochemistry (IHC) analysis

showed increased adhesion molecule expression in the terminal portal venules (TPV) and sinusoids by LPS stimulation. Peak hepatic NO and iNOS induction were detected between 8-12 h by electron paramagnetic resonance (EPR) and IHC. LPS treatment significantly increased melanoma cell retention in the liver (between 8-24 h), especially in the TPVs compared to the controls. By DNA end-labeling, the rates of melanoma cell apoptosis were significantly increased from 8-24 h in the TPV region (but not in the sinusoids) of LPS-treated mice compared with the controls. Fourteen days after tumor cell injection, the LPS-treated mice had a significantly smaller liver surface metastatic tumor burden than the control mice. These data suggest that LPS affects the fate of melanoma cells in the liver by inducing expression of adhesion molecules and production of NO in the hepatic endothelium, and the main action site of LPS is in the TPV region of the liver acinus. Data support the hypothesis that the fate of intravascular metastatic tumor cells can be regulated by modulation of hepatic microvascular functions using an endogenous/exogenous stimulator.

#### #4256 Hirudin Inhibits Human Tumor Implantation and Metastasis in Nude Mice. Merlin Lee, Wendy Campbell, Yao Qi Huang, Roman Perez-Soler, and Simon Karpattin. *New York University School of Medicine, New York, NY.*

The association of thrombosis with cancer is well recognized. Thrombin-treated tumor cells enhance the formation of pulmonary metastasis in mice >100 fold via enhanced adhesion as well as other mechanisms, such as the promotion of angiogenesis. We therefore examined the effect of hirudin, a potent thrombin inhibitor that has the capacity to inactivate adherent thrombin within the tumor thrombus. Nude mice (5 in each group) were injected both subcutaneously and intravenously with  $1 \times 10^6$  human A549 lung cancer cells (non-small cell) as well as breast cancer MDAMB231 cells. Hirudin, 10 mg/kg was given 5 min before and 4 hrs after tumor inoculation and mice followed for various time intervals (16-77 days). Impaired subcutaneous tumor growth was particularly marked with hirudin treatment at early time points following tumor nodule recognition: 3-4 fold less with A549 cells at 12-15 days and 2-3 fold less at 41 days. Similar results were obtained with MDAMB231 cells: 10-11 fold less at 7-9 days and 1.5-3 fold less at 12-16 days. A lag period of 2-5 days was noted before appearance of tumor nodules in animals treated with hirudin compared to controls. Additional hirudin given everyday for 5 days or every other day for 10 days showed no enhancement over the initial 2 injections. When mice injected with MDAMB231 cells were treated with hirudin after the appearance of 0.4cm<sup>2</sup> nodules at 8 days, no difference in tumor growth was noted between treated and control mice at 10 and 14 days. When a group of mice were treated similarly with heparin 80units/kg, no differences in the sizes of the tumors or a lag period was observed compared to the control mice. Animal survival was followed after intravenous injection of A549 cells with and without hirudin, as above. In 4 different experiments all hirudin-treated animals were alive at 38-77 days whereas 60% of control animals were dead in 2 experiments and 100% dead in 2 other experiments. Autopsied lungs of dead animals were loaded with tumor metastasis. In culture, hirudin x 3 days had no effect on growth of MDAMB231 cells x 7 days. Parallel studies were performed on thrombin induction of angiogenesis genes: VEGF and angiopoietin-2 in two different tumor cell lines: prostate DU145 and megakaryocyte CHRF and in primary F54 fibroblasts and endothelial cells (HUVEC). mRNA expression by Northern analysis was enhanced ~5-10 fold and protein synthesis 2-4 fold. Hirudin completely reversed this effect. In addition RT-PCR analysis of VEGF, Ang-1 and Ang-2, capable of distinguishing human from murine host origin in tumor implanted mice, revealed invasion of human tumor cell nodules with murine VEGF, Ang-1 and Ang-2 (not human). Thus, hirudin blocks tumor implantation and pulmonary metastasis (not cell division) which may be related to inhibition of host angiogenesis growth factors.

#### #4257 Strategy to Clone Molecules Mediating Metastasis of Breast Cancer Cells to Bone. Sonoko Narisawa and Jose Luis Millan. *The Burnham Institute, La Jolla, CA.*

More than 70% of breast cancer patients show metastasis to the bone at the time of death. In spite of its clinical importance, the mechanisms that lead to preferential bone metastasis are not well understood. Our work focuses on the initial settlement of metastatic cancer cells in bone. We hypothesize that breast cancer cells released from the primary tumor adhere to stromal and/or endothelial cells in the bone marrow through specific receptor/ligand-like interactions. The human breast cancer cell line, MDA-MB-231 was chosen as our model of bone metastatic breast cancer, since these cells can colonize the bone marrow if injected through the cardiac ventricle in nude mice. Using the "Immortomouse" we established and characterized cell lines that display either bone marrow stromal or endothelial cell phenotypes. One cell line from each group displaying significant binding to the MDA-MB-231 cells were chosen for the development of a binding assay to be used to identify putative homing molecules. We constructed a cDNA library of MDA-MB-231 cells using a mammalian expression vector, pCMV-Script. COS-1 cells transfected with the cDNA library were incubated with the immortalized cell lines and the plasmids were recovered from the bound COS-1 cells. Two cDNAs, A3 and A5, were identified as conferring significantly binding in our assay. Clone A3 encodes a known GPI-anchored cell surface protein not previously described to be involved in bone metastasis. The cDNA sequence of the clone A5 matches a previously reported cDNA sequence but the function of this protein is totally unknown. Northern blot analysis revealed that A3 is expressed in higher amounts in bone metastatic tumor cell lines while A5



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appears to be over-expressed in breast and prostate cancer cell lines irrespective of their metastatic potential. We are currently testing the ability of these two molecules to mediate bone homing *in vivo*.

#### #4258 Identification of the Tumor Cell Surface Receptor for Osteonectin.

Jennifer E. Koblinski, Karin Jacob, and Hynda K. Kleinman. *NIDCR, Bethesda, MD.*

Breast and prostate cancer have a high propensity to metastasize to bone. Morbidity and mortality associated with breast and prostate cancer can be attributed to consequences of bone metastases. We identified osteonectin (OSN, SPARC, BM-40) as a bone factor that can increase invasion of breast and prostate cancer cells while other bone-derived proteins, such as osteopontin and BMP-4, were unable to specifically promote invasion. Matrix metalloproteinase-2 activity was also increased in breast and prostate cancer cell lines, treated with osteonectin. These results suggest that osteonectin binds a cell surface receptor through which it mediates effects on tumor cell invasion and protease activity. Osteonectin affinity chromatography of cell extracts identified a 150 kDa protein that was eluted with EDTA. A similar sized band is observed in osteonectin overlay assays on PC-3 prostate carcinoma cell membrane preparations. Solid-phase binding assays indicate that the binding of breast and prostate carcinoma cells to osteonectin is manganese dependent. Binding to osteonectin is inhibited by RGD but not RGE peptides. These results suggest that the tumor cell surface receptor for osteonectin may be an integrin. Ongoing studies will identify the osteonectin receptor. Identification of the osteonectin receptor in breast and prostate cancer cells may lead to better diagnostic and/or therapeutic approaches for breast and prostate cancer.

#### #4259 Ultra Low Levels of Nitric Oxide Abrogate Hypoxia-Induced Metastatic Phenotypes: Evidence That Reduced Nitric Oxide Mediates a Key Pathway in Oxygen-Regulated Gene Expression.

Charles Henry Graham, Lynne Marie Postovit, Gendie Elizabeth Lash, and Michael Anthony Adams. *Queen's University, Kingston, Canada.*

Experimental and clinical evidence has revealed that lack of oxygen in a tumour is a serious negative prognostic indicator for malignant progression. In particular, hypoxia has been linked to increased metastatic spread. Metastasis involves a series of invasive steps initiated by intravasation of tumour cells within the tumour microcirculation followed by extravasation at a target tissue. The persistent invasion by the tumour cells deeper into the distant tissue appears to be a key activation step in the progress of a secondary tumour. Here we show that, although this metastatic phenotype is markedly upregulated by reducing the levels of oxygen, the causal link may in fact be mediated by downstream reduction in the levels of nitric oxide ("hyponitroxia") and not by a direct effect of hypoxia per se. Our *in vitro* findings, using human breast carcinoma cells (MDA-MB-231), revealed that although hypoxia induces upregulation of urokinase-type plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1) and hyperinvasiveness *in vitro*, these phenotypic alterations are abrogated by treatments with extremely low concentrations of nitric oxide (NO) mimetics (glyceryl trinitrate  $10^{-11}$  M and sodium nitroprusside  $10^{-12}$  M). Using classical pharmacological tools (desferrioxamine, carbon monoxide) to inhibit hemoprotein interactions, we determined that the site of action of the nitric oxide mimetics requires the involvement of a heme containing protein. Finally, using intravenous inoculation of a highly metastatic B16F10 melanoma cell line into C57Bl/6 mice as the *in vivo* model system, we demonstrated unequivocally that ultra-low concentrations of nitric oxide mimetics ( $2 \times 10^{-11}$  M GTN) could completely prevent the hypoxia-induced increase (2.5-fold) in the number of metastatic lung nodules. In fact, the data showed that the nitric oxide mimetic treatment could decrease the metastasis of the melanoma cells to less than 15% of hypoxic controls. These findings indicate that ultra-low dose therapy with NO mimetics may be a new therapeutic strategy to decrease the risk of metastasis associated with tumour hypoxia by preventing or reversing cellular invasiveness. (Supported by the MRC Canada)

#### #4260 Invasion by Metastatic Breast Cancer Cells and by Mammary Epithelial Cells is Induced by the Fibronectin PHSRN Sequence and Requires Interstitial Collagenase Expression.

Donna L. Livant, Kathleen M. Woods Ignatoski, Brian Pilcher, Sonja M. Markwart, and Stephen P. Ethier. *University of Michigan Medical Center, Ann Arbor, MI, and University of Texas Southwestern Medical Center, Dallas, TX.*

Invasion of basement membranes and the interstitial extracellular matrix by cancer cells is required for metastasis, the chief cause of mortality in breast cancer. We explored the roles of the alpha 5 beta 1 and alpha 4 beta 1 integrin fibronectin receptors in regulating invasion by normal mammary epithelial cells, and how the downregulation of alpha 4 beta 1 on metastatic SUM 149 and SUM 52 PE human breast cancer cells caused their constitutive invasiveness in the presence of serum. Since fibronectin is abundant in blood, lymph, and interstitial fluid, its ability to stimulate invasion is crucial for metastasis. Using naturally serum-free SU-ECM basement membranes, we showed that plasma fibronectin accounted for the serum dependence of SUM 149 and SUM 52 PE invasion. Invasion by both cell lines was elicited by the interaction of alpha 5 beta 1 integrin with fibronectin, as well as with a peptide containing the fibronectin PHSRN sequence, Ac-PHSRN-NH<sub>2</sub>. Ac-PHSRN-NH<sub>2</sub> was equally effective at inducing invasion by normal mammary epithelial cells (hmc); however unlike SUM 149 and

SUM 52 PE cells, serum was not sufficient to induce hmc invasion. Because blocking P4C2 anti-alpha 4 beta 1 antibody stimulated hmc invasion of SUM 149 in serum, expression of alpha 4 beta 1 integrin likely prevented serum-induced invasion by these cells. Alpha 5 beta 1-mediated invasion was regulated by alpha 4 beta 1 in hmc because a peptide consisting of the alpha 4 beta 1 sequence, Ac-LHGPSEILDVPST-NH<sub>2</sub>, prevented PHSRN-induced invasion. Blocking body experiments showed that matrix metalloproteinase 1 (MMP-1) was necessary for Ac-PHSRN-NH<sub>2</sub>-induced invasion by SUM 149, SUM 52 PE, and hmc. SUM 52 PE, as well as for anti-alpha 4 beta 1-induced invasion by hmc. Furthermore, MMP-1 expression was shown to be sufficient for invasion induced by Ac-PHSRN-NH<sub>2</sub>, serum, or anti-alpha 4 beta 1 through the use of peptide hydroxamate MMP inhibitors. The broad spectrum inhibitor batimastat inhibited invasion in all cases; whereas, an inhibitor of all MMPs except MMP-1 was permissive.

#### #4261 Expression of FAS Ligand and Metastasis of Colorectal Carcinoma.

Shungo Endo, Hiroyuki Kato, Masahiko Hashimoto, Kazuhiko Yoshimura, Ichiro Ishibashi, Akihiro Umehara, Hajime Yokomizo, Satoshi Kobayashi, Kinoshita, Osamu Watanabe, Shunsuke Haga, and Tetsuro Kajiwara. *Women's Medical University Daini Hospital, Tokyo, Japan.*

[Objectives] Fas ligand (Fas L) expressed on tumor cells has been reported to bind to Fas molecules expressed on immunocompetent cells and induce apoptosis of the latter. Fas L expression by the tumor is, therefore, considered to be related to tumor cell evasion of the host immune response and to metastasis. In view of the potential significance of the presence of Fas L, we examined the relationship between its expression and clinicopathological factors in colorectal carcinomas. [Methods] Subjects were 35 patients after excision of primary colorectal carcinoma. The expression of Fas L was examined in sections of paraffin-embedded specimens by immunostaining with anti-Fas L antibodies. In addition, the expression of Fas L was examined in intrahepatic metastatic foci in 5 cases. The presence of Fas L mRNA was also examined using the RT-PCR method in 14 patients whose cancer tissues had been freshly frozen. Correlations between clinicopathological factors and Fas L expression were examined by the  $\chi^2$  test. [Results] 1) Fas L was expressed strongly in 14 cases, moderately in 7 cases, weakly in 7 cases and absent in 3 cases in primary lesion. Fas positivity was marked in 4 cases and moderate in 1 case of intrahepatic metastatic foci. We classified patients expressing Fas L strongly or moderately as Fas L-positive and those expressing Fas L weakly or not as Fas L-negative. Expression of Fas L mRNA was detected in 12 of 25 cases (48.0%). All of the patients testing positive for Fas L mRNA belonged to the Fas L-positive group. In view of this finding, we performed correlative statistical analyses dividing patients into one Fas L-positive group comprising 25 cases and one Fas L-negative group comprising 10 cases. 3) No correlation was found between the expression of Fas L and the histopathological classification of the tumors, the depth of invasive blood vessel invasion, lymph nodes metastasis or peritoneal dissemination. The frequency of Fas L expression was significantly greater in patients with liver metastasis ( $p=0.0280$ ): 13 of 14 patients with synchronous and metachronous liver metastases were Fas L-positive. 5) In the Fas L-positive group, the frequency of metastasis to the liver was greater in patients with cancers invading veins ( $p=0.0112$ ). [Conclusion] The results of this study suggest that the expression of Fas L in colorectal cancer is related to the implantation and proliferation of cancer cells which have metastasized to the liver.

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#### #4262 Sphingosine-1-Phosphate Inhibits Metastasis and Haptotactic Motility of B16 Melanoma Cells.

Hironori Yamaguchi, Hiroyuki Okamoto, Kitayama, Hirokazu Nagawa, and Yoh Takuwa. *Department of Physiology, Kanazawa University School of Medicine, Kanazawa, Japan, and Department of Surgical Oncology, University of Tokyo Graduate School of Medicine, Tokyo, Japan.*

Objects Sphingosine-1-phosphate (S-1-P), a biologically active lipid mediator has been reported to inhibit the motility of mouse melanoma cells. It is an interesting possibility that S-1-P could inhibit the metastasis of mouse melanoma cells. The purpose of this study is to clarify the effects of S-1-P on pulmonary metastasis *in vivo* of B16 melanoma cells. Methods Effects of S-1-P on haptotactic assay *in vitro*. Four subclones of B16 melanoma cells were selected by limiting dilution. The haptotactic motility of each subclone was assayed using Transwell chambers with filters (8  $\mu$ m pore size). The lower surface of the filter was precoated with fibronectin (5  $\mu$ g/filter) for 3 hours. The cells were suspended by trypsinization at a concentration of  $1 \times 10^6$  cells/ml in DMEM supplemented with 0.1% bovine serum albumin (BSA). Cells (350  $\mu$ l of the suspension) were seeded in the upper compartment. DMEM (100  $\mu$ l) with 0.1% BSA and with  $1 \times 10^{-6}$  M of S-1-P were placed in the lower chamber. After incubation for 3 hours at 37  $^{\circ}$ C, cells on the lower surface of the filter were stained and counted by a spectrophotometer. Effects of S-1-P on pulmonary metastasis *in vivo*. The cells were trypsinized and suspended in Hanks' solution at a density of  $1 \times 10^7$  cells/ml.

**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed for Form Page 2.  
Follow the sample format on for each person. (See attached sample). **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE (ADDRESS/PHONE/EMAIL)		
José Luis Millán	Professor, The Burnham Institute, La Jolla, CA Tel: 858-646-3130; email: millan@burnham.org		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Buenos Aires, Argentina	Clin. Chem	1974	Biochemistry
University of Buenos Aires, Argentina	Biochem.	1976	Biochemistry
University of Umeå, Sweden	Ph.D.	1983	Biochemistry

**A. Positions**

March 1976 - August 1977	Clinical Enzymologist, Clinical Chemistry Institute, Mar del Plata, Argentina.
August 1977 - May 1978	Trainee in Enzymology, La Jolla Cancer Research Foundation, La Jolla, CA
May 1978 - January 1981	Research Associate, La Jolla Cancer Research Foundation, La Jolla, CA
Jan. 1981 - Dec. 1983	Guest researcher, Dept. of Physiological Chemistry, Umeå University, Sweden.
March 1984 - June 1984	Guest researcher, State Serum Institute, Copenhagen, Denmark.
June 1984 - June 1986	Research Associate, La Jolla Cancer Research Foundation, La Jolla, CA
July 1986 - April 1989	Assistant Professor, La Jolla Cancer Research Foundation (The Burnham Institute)
May 1989 - April 1994	Associate Professor, The Burnham Institute, La Jolla, CA.
May 1990 - Aug 1995	Docent (Adjunct Associate Professor) at the University of Umeå, Sweden.
May 1992 - present	Adjunct Professor, University of Murcia, Murcia, Spain.
Sept. 1995 - June 2000	Professor of Medical Genetics, Umeå University, Sweden
July 2000 - present	Adjunct Professor, Umeå University, Umeå, Sweden
May 1994 - present	Professor at the The Burnham Institute, La Jolla, CA

**B. Selected Publications (selected papers from a total of 105 articles)**

Millán, J.L., Whyte, M., Avioli, L. and Fishman, W.H. Hypophosphatasia (adult form) - Quantitation of alkaline phosphatase isoenzymes in a large kindred. Clin. Chem., **26**: 240-245 (1980).

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**Active Research Support**RO1 CA 42595-17  
NIHMillán (PI)  
Direct Costs Year 17: \$162,933

4/1/86 to 6/30/2003

"Cancer-related placental-type alkaline phosphatases".

The aims of this proposal are to elucidate the structure and function of the placental (PLAP) and germ cell (GCAP) alkaline phosphatases during development and the significance of their re-expression in cancer and other diseases.

RO1 DE12889-04  
NIHMillán (PI)  
Direct Costs Year 04: \$191,707

10/1/1998 to 9/30/2003

"Molecular pathogenesis of hypophosphatasia".

This project aims at understanding the enzymatic defects caused by mutations in the TNAP gene and to generate mouse models of adult and hypophosphatasia as well as to attempt correction of the defect by gene therapy using bone marrow mesenchymal stem cells.

RO1 AR 47908-01  
NIHMillán (PI), Dr. Terkeltaub (Consortium)  
Direct Costs Year 1: \$218,250 (\$61,040 earmarked for Dr. Terkeltaub)

4/1/2002 to 3/31/2007

"Counter-regulatory mechanisms in bone mineralization".

This project aims to understand the interplay between TNAP, PC-1 and ANK in controlling intracellular and extracellular pyrophosphate concentrations and thereby regulating bone mineral deposition.

RO1 HD 05863-29  
NIHGoldberg (PI), Millán (Consortium)  
Direct Costs Year 9 of Consortium: \$38,074

7/1/1993 to 6/31/2002

"Reproductive biochemistry of testis-specific LDH-X".

This project aims at elucidating the significance of testis-specific isozyme gene expression by inactivating the mouse *ldh-x* gene and characterizing the resulting defects in spermatogenesis.

**Application submitted for funding**CRP 3IC 0203  
CCRP Cycle IIIMillán (PI)  
Direct Costs requested: \$625,649

7/1/2002 to 6/31/2005

"Diagnostic screening for testicular germ cell tumors".

This application proposes to optimize and use a novel screening technique aimed at detecting the presence of CIS cells in semen samples from patients diagnosed with, or at risk for developing, malignant testicular germ cell tumors. We will also test the hypothesis that detection of gained 12p-sequences, derived from invasive testicular germ cell tumors, present in the serum and/or plasma of testis cancer patients is of clinical relevance.

The proposal was approved, but funding is still pending, subject to California Governor's appropriation of funds for CRP.